

## **SUPPLEMENTAL INFORMATION**

### **Differential Serotonin Transport is Linked to the *rh5-HTTLPR* in Peripheral Blood Cells**

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## SUPPLEMENTAL METHODS AND MATERIALS

**Electrode fabrication.** Electrically conductive boron-doped diamond was deposited over chemically etched platinum wires (25  $\mu\text{m}$  diameter) using microwave-assisted chemical vapor deposition (1.5 kW, 2.54 GHz, ASTeX, Woburn, MA) (1). Boron doping was achieved during the vapor deposition process by adding 10 ppm  $\text{B}_2\text{H}_6$  to 0.5%  $\text{CH}_4/\text{H}_2$ .

**Blood cell preparation.** Peripheral blood cells (PBCs) from rhesus monkeys (*M. mulatta*) were isolated from anti-coagulated blood using a Ficoll gradient centrifugation method. Whole blood samples were diluted with RPMI media (Invitrogen Corporation, Carlsbad, CA). Diluted blood samples were transferred to Ficoll Accuspin tubes and centrifuged at 900 g for 25-30 min at room temperature. The entire top white layer was collected into 5% RPMI media. Samples were centrifuged at 340 g for 10 min. Supernatants were removed and cell pellets were resuspended in 10-20 ml of 10% RPMI. Red blood cells were lysed if detected. Samples were rinsed with fresh 10% RPMI and centrifuged for an additional 10 min at 340 g. The resulting cell pellets were diluted in a solution containing freezing media (RPMI, fetal bovine serum, and dimethyl sulfoxide) and stored at  $-20\text{ }^{\circ}\text{C}$  for 1 h followed by long-term storage at  $-70\text{ }^{\circ}\text{C}$ .

**Cell survival and function.** Frozen samples of PBCs were thawed in assay buffer (150 mM NaCl, 5 mM KCl, 1.2 mM  $\text{MgCl}_2$ , 5 mM glucose, 10 mM HEPES, 2 mM  $\text{CaCl}_2$  at pH 7.4). Cell type and viability were analyzed by flow cytometry (FC 500, Beckman Coulter, Miami, FL) using propidium iodide (PI) to identify dead cells. Forward and side scatter plots indicated that cell populations were predominantly mononuclear cells, *e.g.*, B- and T- lymphocytes,

and some platelets. (Fig. S2). Cell survival after thawing was investigated with respect to assay buffer oxygenation, holding temperature, and holding time.

**Electrochemistry.** High-speed chronoamperometry using boron-doped diamond electrodes was carried out as described previously (2). All experiments were performed in 12-well polystyrene plates (BD Biosciences, San Jose, CA). Reference and working electrodes were carefully lowered into wells containing assay buffer alone or assay buffer containing PBCs, and background currents were monitored. The data for electrode calibration and serotonin uptake experiments were analyzed according to previously reported methods (2). Electrodes were calibrated prior to each experiment against known concentrations of serotonin in assay buffer (0.1-1  $\mu$ M), and responses were analyzed for linearity. Electrodes were re-calibrated after each experiment to measure changes in electrode sensitivity due to fouling. Pre- and post-calibration values were averaged and used to estimate analyte concentrations.

**Serotonin uptake.** Frozen PBCs (~10 million cells/ml) were thawed by adding 12-15 ml of room temperature assay buffer. A small volume (200  $\mu$ l) of PBCs was used for counting live cells using Trypan blue exclusion. On average, 75% of the cells survived the freezing and thawing procedure. Peripheral blood cells were then centrifuged at 340 g for 7 min. Pellets containing PBCs were resuspended by gently vortexing in assay buffer to produce final concentrations of 2-4 million cells/ml. Solutions of PBCs were maintained at 4 °C for no more than 4 h before uptake experiments.

Immediately prior to measuring serotonin uptake, PBCs were centrifuged at 340 g for 7 min. Fresh assay buffer saturated with 95% O<sub>2</sub>/5% CO<sub>2</sub> by bubbling the gas mixture for at least 30 min was utilized at room temperature for all experiments. Cells were resuspended in oxygenated assay buffer by gentle vortexing. Monoamine uptake by plasma membrane transporters requires coupling to the Na<sup>+</sup>/K<sup>+</sup>-ATPase, which is dependent on oxidative respiration to provide high levels of ATP. Therefore, in some experiments, frozen PBCs were thawed and resuspended in non-oxygenated assay buffer to investigate the effects of oxygen on serotonin uptake. In other experiments, PBCs were pre-incubated for 45 min with 100 nM nisoxetine (NET inhibitor), 300 nM mazindol (NET and DAT inhibitor) (3), or 1 μM decynium-22 (OCT 3 inhibitor) (4) in assay buffer to investigate the specificity of uptake in the presence of inhibitors for other transporters.

One sample of PBCs from each animal was analyzed for total protein content per million cells using the Lowry assay (5). Serotonin uptake rates, RTI-55 binding, and neurochemical data were calculated per million cells vs per mg protein and compared, as shown in Table S3.

**Surface SERT binding.** For specific binding, PBC pellets were resuspended by gently vortexing in binding buffer (10 mM Tris-HCl, pH 9.5, containing 300 mM NaCl and 1 mM MgSO<sub>4</sub>) with different concentrations of cold ligand RTI-55 (1-100 fmol), hot ligand [<sup>125</sup>I]RTI-55 (4.4-15 fmol; 0.0011-0.0032 μCi) and 10 μM GBR12935 to inhibit RTI-55 binding to DAT. For nonspecific binding, 5 μM paroxetine was added to block RTI-55 binding to SERT. For nonspecific and specific binding, incubations were carried out in triplicate in final volumes of 100 μl. Binding was terminated using ice-cold stop buffer

(10 mM Tris-HCl, 200 mM NaCl, pH 7.5) followed by filtration of cell suspensions using a cell harvester (Brandel, Gaithersburg, MD) and GF/F glass fiber filters (0.7  $\mu$ m pore size) presoaked in 0.3% polyethylamine. Filter papers were washed three times with ice-cold stop buffer. Radioactivity associated with each filter/sample was measured using a gamma counter. Binding data are expressed as fmol/million cells or fmol/mg protein.

**Total RNA isolation and cDNA preparation.** Peripheral blood cells (~8-9 million cells per sample) were thawed at room temperature, diluted with 4 ml of assay buffer, and centrifuged at 4 °C for 10 min at 340 g. Supernatants were removed and 1 ml Qiazol® lysing reagent (Qiagen, Huntsville, AL) was added to the cell pellets. Samples were vortexed and passed through a 23 gauge needle ~10-12 times. Phase separation was achieved by adding 200  $\mu$ l of isoamyl/chloroform, and the aqueous layer containing total RNA was removed. One volume of isopropanol and 150  $\mu$ g/ml GlycoBlue (Ambion, Austin, TX) were added and the samples were stored overnight at -20 °C for precipitation. Samples were washed with 1 ml of 75% ethanol and RNA pellets were dried for 10 min before resuspending in 80  $\mu$ l of nuclease-free water. A NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE) was used to assess the concentration and purity (A260:A280) of the RNA samples. Subsequently, 5  $\mu$ g of RNA from each sample was subjected to DNase treatment (Ambion Turbo DNA-free Kit, Austin, TX) following the manufacturer's protocol to eliminate genomic DNA. Samples were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit and MultiScribe™ Reverse Transcriptase (Applied Biosystems, Foster City, CA) as per the manufacturer's instructions.

**Real time-qPCR.** Real time quantitative PCR (RT-qPCR) and TaqMan probes and primers specific to *SERT* and two control genes, *ACTB* and *GAPDH*, were used for amplifications. Amplifications of cDNA from individual genotyped rhesus PBC samples were carried out using 80 ng cDNA, primers (900 nM), probes (250 nM), and Fast Universal PCR Mastermix (Applied Biosystems, Foster City, CA) in quadruplicate in 15 µl reaction volumes using an ABI 7900HT Fast Real Time PCR System (Applied Biosystems). Relative quantification was determined by subtracting the cycle threshold (Ct) of the geometric mean of the two control genes combined across samples from the cycle threshold values for *SERT* ( $\Delta Ct$ ). Values were transformed exponentially using the equation  $2^{-\Delta Ct}$ .

Primers and probes were validated by amplifying serial dilutions of cDNA from pooled rhesus PBCs. A semi-log plot of [cDNA] versus cycle threshold values at each dilution was used for linear regression analyses to obtain the efficiency (E) of the primer/probe sets ( $E = (10^{-1/\text{slope}}) * 100$ ). Amplification efficiencies for *SERT*, *GAPDH*, and *ACTB* were 97%, 98%, and 82%, respectively. Sequences and references for the primers and probes are found in Table S3.

**Neurotransmitter and metabolite measurements.** Thawed PBCs (~10 million cells/ml) were centrifuged at 340 g for 7 min. Pellets containing PBCs were resuspended by vortexing in 0.1 M HClO<sub>4</sub> with *N*-ω-methyl-5-hydroxytryptamine (N-Me-5HT; 250 nM final concentration) added as the internal standard in a final volume of 1.5 ml. Peripheral blood cells solutions were sonicated using a probe sonicator and 12-15 one-second pulses to disrupt membranes and other large biomolecules. Cell homogenates were centrifuged at 12,000 g for 2 min and 150 µl of each supernatant was stored at -70 °C until analysis.

Supernatants were analyzed by high performance liquid chromatography with electrochemical detection. After thawing, supernatants were centrifuged at  $16,000 \times g$  for 10 min at  $4^{\circ}\text{C}$ . Separation was carried out by flowing mobile phase containing 9% acetonitrile, 0.35 g/L octanesulfonic acid,  $10 \mu\text{M}$  EDTA, and 0.1% triethylamine in 0.1 M monochloroacetic acid at  $230 \mu\text{L}/\text{min}$  through a  $150 \text{ mm} \times 2 \text{ mm}$  column containing  $3 \mu\text{m}$  C18 particles as the stationary phase (Phenomenex, Torrance, CA). Serotonin and its major metabolite, 5-hydroxyindoleacetic acid (5-HIAA) were detected by a graphite-working electrode (WE-3G) at  $+700 \text{ mV}$  vs Ag/AgCl on an HTEC 500 HPLC instrument (Eicom, San Diego, CA). Relative peak areas were quantified using PowerChrom software (eDAQ, Colorado Springs, CO). Data are expressed as ng neurotransmitter/million cells or ng/mg protein.

**Chemicals.** Serotonin, N-Me-5HT, paroxetine, nisoxetine, mazindol, decynium 22, and chemicals for lymphocyte isolation were purchased from Sigma-Aldrich (St. Louis, MO). Nafion was purchased from Ion Power (New Castle, DE).  $(-)-2\beta\text{-Carbomethoxy-3}\beta\text{-(4-iodophenyl)tropane}$  (RTI-55) was obtained from the Research Triangle Institute International (Research Triangle Park, NC) (6).  $[^{125}\text{I}]$ RTI-55 was purchased from Perkin Elmer (Waltham, MA). All chemicals used for buffer preparations were from VWR (West Chester, PA).

**Data analysis and statistics.** For chronoamperometry experiments, initial uptake rate calculations were performed as previously described (2). Initial serotonin uptake rates for various concentrations of serotonin were plotted, and maximal uptake rates and affinity

constants for data from each animal were calculated using non-linear curve fitting to the Michaelis-Menten equation.

For surface SERT expression, specific radiochemical binding was calculated by subtracting nonspecific binding from total binding for replicate means at each ligand concentration. Specific binding was plotted vs ligand concentrations. Maximal surface binding and dissociation constants for data from each animal were calculated using non-linear curve fitting to a one-site specific binding function.

Data comparing two means for serotonin uptake, SERT binding, and SERT mRNA levels were analyzed using one-tailed unpaired Student's *t*-tests based on *a priori* hypotheses about the direction of change associated with the *rh5-HTTLPR* 'S' allele. Data comparing two means for neurochemicals were analyzed using two-tailed unpaired *t*-tests because no *a priori* hypotheses about the direction of change were made. One-way analysis of variance (ANOVA) was used in cases where more than two means were compared. For maximal serotonin uptake rates, *post hoc* analysis was performed using *a priori* one-tailed Student's *t*-tests to compare *L/L* vs *S/L* or *L/L* vs *S/S* groups. For neurochemical analysis, *post hoc* analysis was performed using Dunnett's multiple comparisons tests. Values that differed by more than  $\pm 3$  SDs from the means were considered outliers and omitted from the final analysis (one point, Fig. 4). Data in Figure S1 were analyzed by two-way ANOVA with repeated measures on time. Curve fitting and statistical analyses were performed using GraphPad Prism v.4 for Mac (GraphPad Software, La Jolla, CA).

## SUPPLEMENTAL RESULTS

**Peripheral blood cells retain viability and SERT function after thawing.** Cell survival rates were assessed with respect to holding temperature, time, and assay buffer oxygenation using flow cytometry with propidium iodide (PI) to stain dead cells. We observed that keeping cells at 37 °C reduced viability compared to holding at 4 °C (Fig. S1). Based on these findings, all subsequent experiments were performed in PBCs held at 4 °C in non-oxygenated assay buffer for no longer than 4 h prior to the start of each experiment.

The compound IDT307, a substrate for monoamine transporters including SERT, fluoresces only after being transported into cells (7, 8). Here, we used IDT307 to investigate whether PBCs were functional after thawing using flow cytometry. Double labeling with 50  $\mu$ l of 1 mg/ml PI solution and 1  $\mu$ M IDT307 was used to subtract fluorescence associated with nonspecific uptake by dead cells. After 15 min of incubation with IDT307 at room temperature in oxygenated assay buffer, PBCs showed a >2-fold increase in fluorescence intensity suggesting active uptake of IDT307 by live PBCs (Fig. S2C). These experiments demonstrate that PBCs can be stored at -70°C for at least one year and still retain viability and SERT function after thawing.

## SUPPLEMENTAL REFERENCES

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## SUPPLEMENTAL TABLES AND FIGURES

ID	Origin	Sex	DOB	Age (yr)	Weight (Kg)	Tph2-1 T to G	Tph2-2 G to A	Tph2-3 A to G	Tph2-4 A to C	MAO-A 5/6/7 repeats	rh5-HTTLPR s/lxl
1	Ch	M	7/15/01	6.15	8.4	ND	G/G	A/A	A/A	8/8	L/L
2	US	M	10/28/00	6.87	7.6	T/T	G/G	A/A	A/A	6/6	L/L
3	US	M	9/16/00	6.98	8.1	T/T	G/G	A/A	A/A	7/7	L/L
4	US	M	5/27/01	6.29	7.6	T/T	G/G	A/A	A/A	6/6	L/L
5	US	M	10/30/00	6.86	9.1	T/T	G/G	A/G	A/A	8/8	L/L
6	US	M	2/11/00	7.58	9.8	T/T	G/G	A/A	A/A	7/7	L/L
7	Ch	M	2/13/00	7.57	7.9	T/T	G/G	A/A	A/A	6/6	S/L
8	Ch	M	5/26/01	6.29	10.6	T/T	G/G	A/A	A/A	7/7	S/L
9	US	M	7/26/00	7.12	8.4	T/T	G/G	A/A	A/A	ND	S/L
10	Ch	M	2/14/00	7.57	7.8	T/T	G/G	A/A	A/A	7/7	S/S
11	Ch	M	5/17/00	7.32	7.9	ND	A/G	A/A	A/A	7/7	S/S
12	Ch	M	1/1/00	7.69	7.2	T/T	G/G	A/A	A/A	7/7	S/S
13	US	F	10/31/00	6.86	4.2	T/T	G/G	A/G	ND	6/7	S/S
14	US	F	2/23/01	6.54	6.0	T/T	G/G	A/A	A/A	6/7	S/S
15	US	F	1/2/01	6.68	6.2	G/T	G/G	A/A	A/A	7/7	S/S
<b>Average</b>				<b>7.0</b>	<b>7.8</b>						
<b>SEM</b>				<b>0.1</b>	<b>0.4</b>						

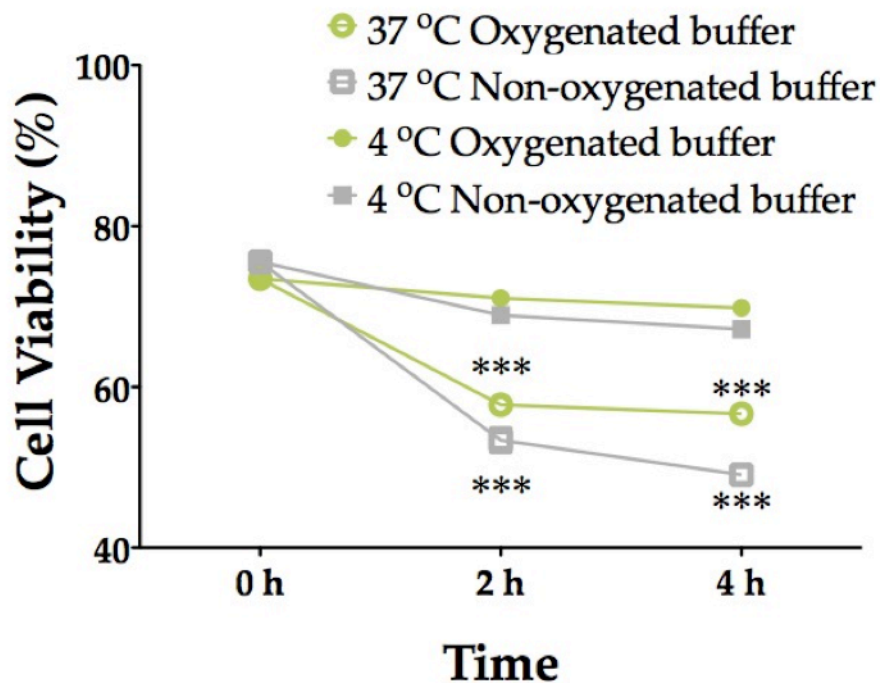
**Table S1. Cohort Characteristics.** Animals born in China are indicated by “Ch” while animals born in the United States are indicated by “US”. Ages and weights for animals were recorded at the time of initial blood withdrawal for genotyping. Genotypes for polymorphisms in the tryptophan hydroxylase-2, monoamine oxidase type-A, and serotonin transporter genes are shown for the cohort studied. Samples where genotype could not be determined are labeled “ND”.

Gene	Reference		Sequence	GenBank Accession #	Efficiency
<i>SERT</i>	Yu et al. 2010	Forward	5' CTCTTGGTCGGGCTTTGG 3'	AF285761.1	0.97
		Reverse	5' TGCTCACCAGGGGCATCTTG 3'		
		Probe	5' FAM-TTTGCTAGCTACAACAAGTTCAACAACACTGCTAC-TAMRA 3'		
<i>ACTB</i>	Kinnally et al. 2010	Forward	5' TGAGCGCGGCTACAGCTT 3'	NM001033084.1	0.82
		Reverse	5' CCTTAATGTACGCACCGATT 3'		
		Probe	5' FAM - ACCACACGGCGGAGCGG - TAMRA 3'		
<i>GAPDH</i>	Yu et al. 2010	Forward	5' GACCAC AGTCCATGCCATCA 3'	DQ894744.1	0.98
		Reverse	5' CATCAGCCACAGTTTCC C 3'		
		Probe	5' FAM - ACCCAGAAGACTGTGATGGCCCC - TAMRA 3'		

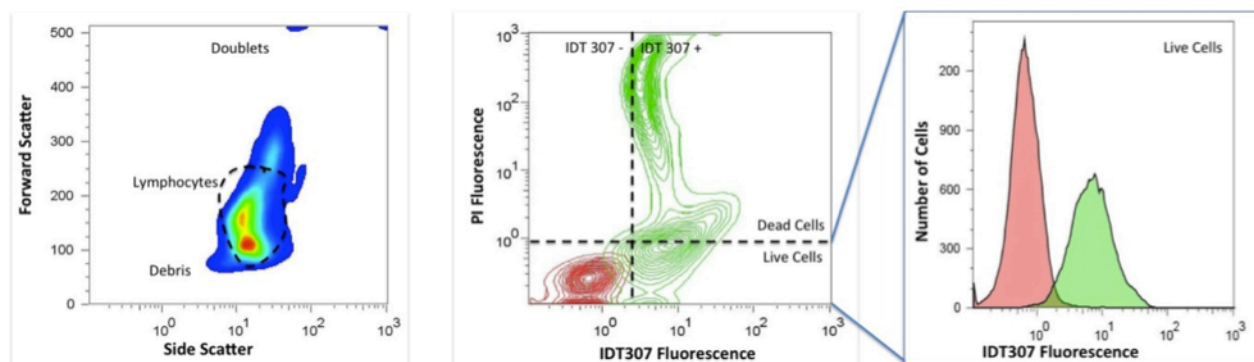
**Table S2. Sequences of primers and probes for RT-qPCR.** To investigate changes in SERT mRNA levels in rhesus peripheral blood cells, real-time quantitative PCR (RT-qPCR) and TaqMan probes and primers specific to *SERT* and two control genes, *ACTB* ( $\beta$ -actin) and *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase), were used for amplifications.

SERT Uptake Analysis	Maximal Uptake Rates				Affinity Constant	
		(pmol /million cells-min)		(pmol /mg protein-min)	(μM)	
	L/L-genotype	7.1 ± 0.4		35 ± 2	0.45 ± 0.06	
	S/L-genotype	5.1 ± 0.2**		24 ± 3**	0.39 ± 0.05	
	S/S-genotype	4.9 ± 0.6**		25 ± 3**	0.45 ± 0.1	
	[F(2,12)=5.9; P<0.05]		[F(2,12)=4.7; P<0.03]	[F(2,12)=0.086; P<0.92]		
SERT Binding Analysis	Maximal Binding Rates				Binding Constant	
		(fmol /million cells)		(fmol /mg protein)	(nM)	
	L/L-genotype	3.5 ± 0.4		17 ± 2	0.27 ± 0.04	
	S/L-genotype	2.4 ± 0.4		12 ± 3	0.29 ± 0.08	
	S/S-genotype	2.6 ± 0.4		13 ± 2	0.26 ± 0.07	
	[F(2,12)=2.5; P<0.12]		[F(2,12)=1.3; P<0.3]	[F(2,12)=0.054; P<0.95]		
Neurochemical Analysis	Serotonin		5-HIAA		5-HIAA/5-HT	
		(ng /million cells)	(ng /mg protein)	(ng /million cells)	(ng /mg protein)	
	L/L-genotype	0.25 ± 0.08	1.2 ± 0.4	0.014 ± 0.002	0.067 ± 0.01	0.014 ± 0.002
	S/L-genotype	0.16 ± 0.04	0.82 ± 0.3	0.006 ± 0.001*	0.029 ± 0.01*	0.009 ± 0.002
	S/S-genotype	0.23 ± 0.03	1.2 ± 0.2	0.008 ± 0.002*	0.042 ± 0.01*	0.006 ± 0.001*
	[F(2,10)=0.55; P<0.59]	[F(2,10)=0.53; P<0.61]	[F(2,10)=6.9; P<0.013]	[F(2,10)=6.3; P<0.017]	[F(2,10)=5.5; P<0.05]	

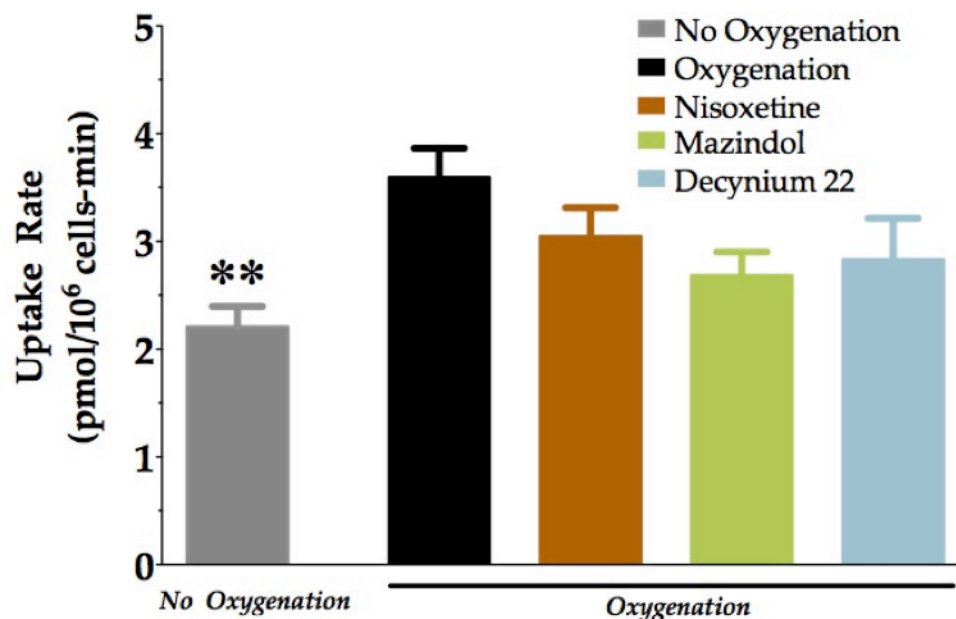
**Table S3.** Summary of chronoamperometric SERT uptake data, radiometric surface SERT binding assay, and HPLC neurochemical analysis are shown for means  $\pm$  SEMs. \* $P$ <0.05, \*\* $P$ <0.01 vs the L/L genotype. Data were analyzed per million cells or per mg protein with essentially the same results.



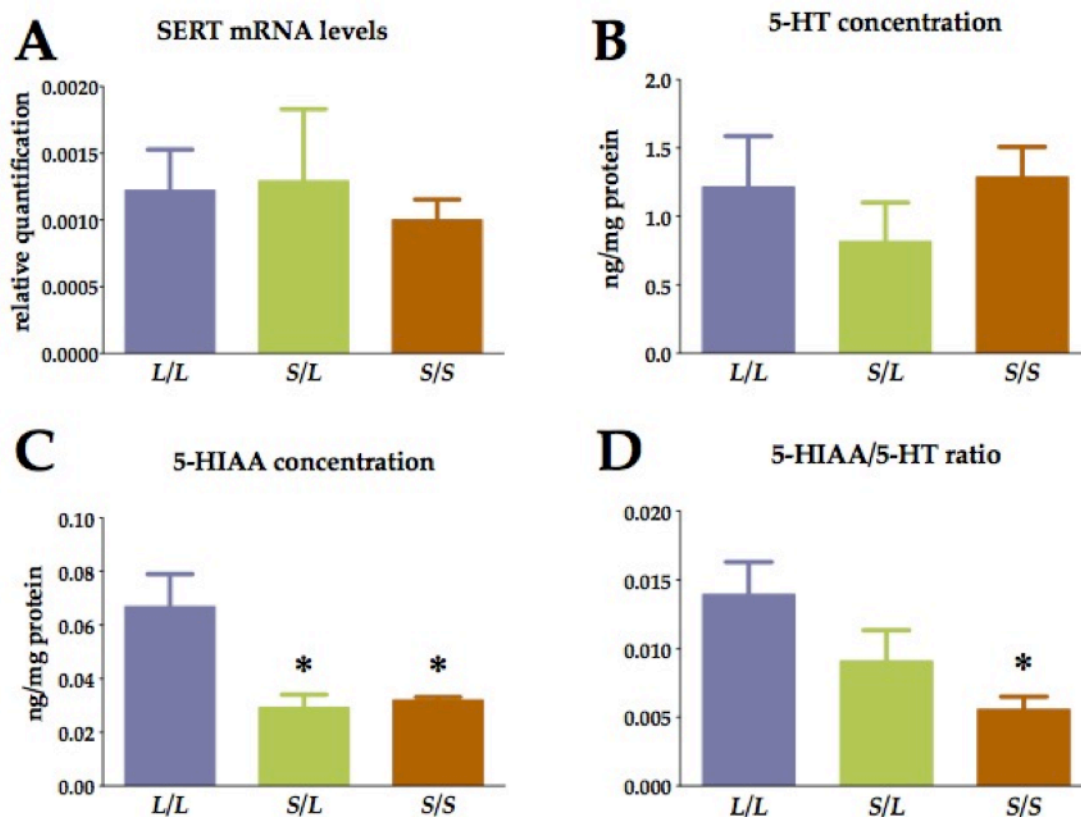
**Figure S1. Cell viability depends on holding temperature.** Typically, each uptake experiment requires 2-4 h to perform. Thus, cell viability over this time frame was investigated using propidium iodide to stain dead cells in combination with flow cytometry. Freshly thawed PBCs ( $t = 0$  h) showed ~25% cell death. Further decreases in cell viability were not observed even after 4 h at 4 °C. By contrast, holding PBCs at 37 °C leads to additional reductions in cell viability. Oxygenation of the assay buffer did not alter cell survival at either holding temperature. Data are means of percent cell viability  $\pm$  SEMs ( $N=4$  samples per data point). \*\*\* $P<0.001$  vs 0 h.



**Figure S2. Flow cytometry in rhesus peripheral blood cells.** A representative forward vs side scatter plot is shown in the left panel providing information about the size and complexity of the cell samples, respectively. The populations of cells that are characteristic of PBCs were identified from this plot. In the middle panel, a fluorescence plot of PBCs before (red) and after (green) incubation with propidium iodide (PI) and IDT307, a fluorescent serotonin transporter substrate. Fluorescence intensity is shown for IDT307 on the x-axis and PI on the y-axis. Propidium iodide was used as an indicator of dead cells. The dotted line parallel to the x-axis indicates the cut off for live cells as determined by the background fluorescence intensity of PBCs in the absence of PI. The histogram in the right panel shows an order of magnitude increase in the fluorescence intensity of live cells after exposure to 1  $\mu$ M IDT307 for 15 min (green curve) vs at the beginning of the incubation period (red curve) suggesting that previously frozen PBCs have functional serotonin transporters.



**Figure S3. Characteristics of serotonin uptake by rhesus peripheral blood cells.** Serotonin clearance by rhesus peripheral blood cells (PBCs) was investigated using chronoamperometry with boron-doped diamond microelectrodes. Uptake rates under various conditions are shown as means  $\pm$  SEMs for  $N=3-6$  samples per group. One-way ANOVA showed a significant effect of treatment on uptake rates [ $F(4,17)=4.6$ ;  $P<0.05$ ]. *Post hoc* Dunnett's comparisons to oxygenated samples (black bar) showed that uptake rates were significantly decreased in the absence of assay buffer oxygenation. No significant differences in uptake rates were observed after incubation with the norepinephrine transporter (NET) inhibitor nisoxetine, the mixed NET and dopamine transporter inhibitor mazindol or the organic cation transporter-3 inhibitor decynium 22. \*\* $P<0.01$  vs oxygenation/no drug.



**Figure S4. Characteristics of the serotonin system in peripheral blood cells with respect to the *rh5-HTTLPR*.** **(A)** Real time qPCR was used to measure relative levels of SERT mRNA in rhesus blood cells. No differences in SERT mRNA with respect to *rh5-HTTLPR* genotype were detected [ $F(2,11)=0.27$ ;  $P<0.76$ ]. **(B)** Serotonin concentrations were measured by high performance liquid chromatography in peripheral blood cells. One-way ANOVA showed that serotonin levels were not different with respect to genotype [ $F(2,10)=0.53$ ;  $P<0.60$ ]. **(C)** By contrast, 5-HIAA levels were significantly different with respect to genotype [ $F(2,10)=6.3$ ;  $P<0.017$ ]. **(D)** The 5-HIAA to 5-HT ratio also showed a significant effect of genotype [ $F(2,10)=4.1$ ;  $P<0.049$ ]. *Post-hoc* Dunnett's comparisons against the L/L genotype showed significant reductions in 5-HIAA levels in S/L and S/S groups and the 5-HIAA to 5-HT ratio for the S/S group. Data are means  $\pm$  SEMs with  $N=5-6$  for L/L,  $N=3$  for S/L and  $N=5-6$  for S/S groups. \* $P<0.05$  vs the L/L genotype.